ISOLATED AND RECOMBINANT ANTIMICROBIAL PEPTIDES THROMBOCIDIN-1 (TC-1) AND THROMBOCIDIN-2 (TC-2) OR VARIANTS THEREOF

The present invention relates to isolated antimicrobial peptides TC-1 and TC-2 or variants thereof and to recombinantly prepared TC-1 and TC-2 or variants thereof.

Antibiotics are commonly used in the treatment and/or prevention of infectious diseases caused by various microorganisms. However, resistance of bacteria against these antibiotics often occurs. Thus, methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-resistant Staphylococcus epidermis (MRSE) are well-known resistant microorganisms, among other bacterial species, responsible for serious nosocomial infections that are very difficult to treat.

Conventional antibiotics kill bacteria by binding to specific targets that are involved in bacterial DNA and protein synthesis or in cell wand synthesis. Resistance can occur when bacteria modify these targets, such that antibiotics do not bind to these proteins, or when bacteria produce specific enzymes that inactivate the antibiotics. Glycopeptide antibiotics such as vancomycin can be used to treat these resistant microorganisms, but their use must be limited to prevent development of resistance to these "kill or cure" remedies as well.

As more and more bacteria become resistant to routinely applied antibiotics, there is an increasingly urgent need for alternative antibacterial agents.

The object of the present invention is therefore to provide new antimicrobial agents against which microorganisms do not rapidly become resistant.

~~This object is achieved by the invention by providing new, isolated or recombinant, antimicrobial peptides thrombocidin-1 (TC-1) and thrombocidin-2 (TC-2),~~

~~or variants thereof, such as TC-1*, which comprise, at~~ least in part, the sequence as shown in figure 1 ~~and~~ have broad antimicrobial activity. These peptides, or variants thereof, thus may be ~~effectively~~ used as antibiotics in the treatment of ~~several~~ infectious diseases. These peptides ~~can~~ be isolated from both human and animal ~~tissue.~~

"Variants" of isolated or recombinant peptides TC-1 and TC-2 are peptides that are at least 70% homologous, preferably at least 80%, more preferably at least 90%, most preferably at least 95%, to TC-1 and TC-2 and also have antimicrobial activity in vitro, such as TC-1*.

It has been found that human and animal blood platelets contain factors that exhibit antibacterial and antifungal activity in vitro. Upon thrombin-activation platelets are known to release lysozyme, as well as a number of other cationic peptides. A number of these platelet-derived peptides have been identified, such as platelet factor-4 (PF-4), RANTES, connective tissue activating peptide (CTAP-III), platelet basic protein (PBP), and neutrophil activating peptide (NAP-2).

In the research that led to the invention, new peptides have been isolated from platelet granules, and have been purified and characterized. These peptides are small and strongly positively charged proteins and are named thrombocidins (TC). The positive charge of thrombocidins presumably accounts for their antibacterial activity. Thus, like other cationic antibacterial proteins, thrombocidins most likely form pores in bacterial membranes, as a result of which these bacteria will die. Since thrombocidins act on the bacterial membrane itself which can not easily be modified, resistance will not rapidly occur.

Thrombocidins are stored in alpha-granules of platelets and are released following thrombin-activation. The most active thrombocidins, TC-1 and TC-2 and variants, such as TC-1*, have been isolated, purified and

characterized as described in further detail in the following examples. In short, thrombocidins were isolated from a large batch of human platelets. The first step was to isolate the platelet granules in which the thrombocidins are present. These platelet granules subsequently were disrupted and the protein content was collected for further purification. The thrombocidins were separated from other proteins on the basis of molecular size, polarity and charge.

~~The new peptides of the invention appear to be~~ derivatives of NAP-2 and CTAP-III. NAP-2 itself ~~is~~ a N-terminal cleavage product of CTAP-III. TC-1 has been shown to be a mixture of C-terminal truncation products of NAP-2, of which the 7436 Da peptide, lacking two C-terminal amino acids, is the main component (referred to as variant TC-1*; figure 1, table 1). A form of NAP-2 with an additional N-terminal tyrosine was also present as a minor component. TC-2 has been identified as a C-terminal truncation product of CTAP-III lacking the last two C-terminal amino acids, with a molecular weight of 9100 (figure 1A, table 1). Thrombocidins identified thus far are indicated in fig. 1A, together with the known ~~sequences of CTAP-III and NAP-2 (fig. 1);~~

As described earlier, the thrombocidins are C-terminal truncation products of NAP-2 and CTAP-III, both of which are chemokines of the CXC family. These chemokines have a specific arrangement and disulfide-linkage of 4 cystein residues, giving the molecule a characteristic three dimensional structure (fig 13). Therefore it is well possible that other chemokines or chemokine-like molecules possess antimicrobial activity like the thrombocidins.

Based on the amino acid sequence of the native proteins the DNA coding for TC-1, NAP-2 and CTAP-III were now cloned and used to recombinantly produce these proteins in order to obtain larger quantities. Hereto, coding DNA was amplified from a human bone marrow cDNA library by PCR and cloned into a NdeI/BamHI digested

pET9a or pET16b expression vector. Constructs were transformed to E. coli BL21DE3LysS cells and gene expression was induced by adding IPTG to growing cultures. Bacteria were harvested, lysed in guanidine, and recombinant proteins were purified to homogeneity in a two-step purification. Sequences of the purified recombinant proteins are shown in figure 2. Recombinant thrombocidins were also produced in the milk of animals.

Several classes of antibacterial proteins contain disulfide bonds. As far as it has been investigated, the presence of these disulfide bonds has been found to be essential for antibacterial activity of HNP-2 (Selsted and Harwig, 1989), GNCPs (Yomogida et al, 1995) and beneficial for activity of protegrins (Harwig et al, 1996). Because disulfide formation is critical for antibacterial activity, a prokaryotic system is not an obvious way to produce these proteins recombinantly. HNP has been produced recombinantly in E. coli, but indeed this product had no antibacterial activity, probably due to misfolding of the protein (Piers et al, 1993). Chemokines like NAP-2, CTAP-III (Proudfoot et al, 1997) and IL-8 (Lindley et al, 1988) have been produced in E. coli, but these proteins had to be refolded after they had been purified, a procedure which was not needed to observe antibacterial activity of the recombinant thrombocidins.

Bactericidal activity of TC-1* and TC-2 has been confirmed against a number of gram-positive, such as Streptococcus sanguis, Staphylococcus aureus, Staphylococcus epidermidis, and Bacillus subtilis and gram-negative bacteria such as Escherichia coli, which are found in a wide variety of infections.

One example of an infection in which the peptides of the invention may be used is endocarditis. Endocarditis is a serious infectious heart disease with high morbidity and mortality, associated with abnormalities of the heart endothelium or the heart valves. Lesions due to these abnormalities give rise to

platelet adherence and activation. Together with other blood proteins, these platelets form a dense meshwork covering the lesion, to which bacteria that have entered the blood stream will adhere. In turn, more platelets will adhere to the bacteria and the 'clot' will grow, ultimately causing malfunctioning of the valve resulting in the need for valve replacement. S. sanguis is one of the microorganism prevalent in native valve endocarditis. Other bacterial, or fungal microorganisms may, however, also be found.

In the platelet clot, bacteria are protected from phagocytic cells, which cannot penetrate the dense platelet meshwork. In contrast, platelet-derived thrombocidins are capable of penetrating the clot and thus may effectively prevent bacterial proliferation.

Fungi against which the peptides of the invention may be effective comprise, for example: Candida albicans, C. glabrata, Cryptococcus neoformans, Aspergillus flavus, A. fumigatus, and Pseudoallescheria spec..

~~The present invention thus provides new,~~ isolated or recombinantly prepared peptides TC-1 and TC-2, or variants thereof, such as ~~TC-1~~ (fig 1 and 2), which exhibit antibacterial and/or antifungal activity and can be used in the treatment of infections in humans and animals. Furthermore, the peptides, or variants thereof, of the present invention can be used for the preparation of a medicament for the treatment of ~~bacterial and/or fungal infections~~.

The invention is further illustrated, but not limited by the following examples and figures.

EXAMPLES

EXAMPLE 1

Isolation, purification and characterization of TC-1 and TC-2

A. Isolation of granule protein from thrombocytes

Buffy coats of human blood from healthy subjects were obtained from the Central Laboratory for Bloodtransfusion, Amsterdam, The Netherlands. Eight buffy coats were pooled in a transfer bag (NPBI, Emmer-Compascuum, The Netherlands) (ca. 550 ml), and 200 ml of PBS + 0.38% tri-sodium citrate (w/v) was added. The bag was blown tight with air and centrifuged for 5 min at 600 g and 20°C. The upper phase, containing mainly platelets, was transferred to a new transfer bag. To this platelet concentrate, 1/9 volume of citrate solution was added (75 mM trisodium citrate; 38 mM citric acid). The bag was blown tight again, and was centrifuged for 10 min at 1750 g (20°C) to pellet platelets. Platelets were resuspended in the same bag in Tris-citrate (63 mM Tris-HCl; 95 mM NaCl; 5 mM KCl; 5mM EDTA; pH 6.8) by gentle massage, and kept shaking overnight at 22°C. Then, platelets were collected in a siliconized flask and the transfer bag was washed with Tris-citrate. Processing of 48 buffy coats routinely yielded ca. 75 ml of highly concentrated platelet suspension containing <0.05% residual leukocytes.

To isolate platelet granules, the platelet concentrate was cavitated three times under nitrogen at 60 atm in a Parr cavitation chamber, and cavitate was collected in siliconized 50 ml tubes (Falcon). This resulted in ca. 90% homogenization of the platelets as determined by Coulter counting. Intact platelets and platelet ghosts were removed by centrifuging the cavitate at 5000 g for 20 min. The supernatant was collected and centrifuged at 12000 g for 20 min, yielding the granules in the pellet. The pellet was resuspended in 5% acetic acid, and sonicated for 30 seconds (pulsed) on ice to

rupture granules. The sonicate was kept at 4°C for 24 hours, and subsequently was centrifuged at 125000 g. The supernatant containing granule protein was dialyzed against 5% acetic acid.

B. Purification of thrombocidin-1 and -2

A rapid two-step purification protocol was developed for the purification of TC-1 and TC-2 from platelet granule protein: i) cation exchange chromatography, and ii) preparative acid urea polyacrylamide gel chromatography (AU-PAGE), to yield highly purified protein preparations.

i) Cation exchange chromatography

As an ion exchange matrix, CM-sepharose 25 (Pharmacia) was used; phosphate buffer (50 mM, pH 7.0) was used as the mobile phase. A 25 ml sample, containing 3.5 mg/ml of granule protein from approximately 40 buffy coats, was loaded at 0.8 ml/min. Subsequently, the column was washed with phosphate buffer, and protein was eluted with a salt gradient from 0 to 1 M NaCl. Fractions were collected, dialyzed and assayed for the presence of antibacterial proteins by running two separate acid urea gels in parallel. One gel was silverstained (fig 3), the other gel was used in an overlay assay to detect antibacterial activity (fig 4). E. coli ML35 was used as the test organism. Selected fractions were analyzed by tricine gel electrophoresis to estimate molecular weights of the (partially) purified protein (fig 5). The activity present in the starting material (fig 3, cav) is eluted in fractions 35 through 75. Major antibacterial activity can be assigned to two proteins, the most cationic protein is designated as thrombocidin-1 (TC-1), the slightly less cationic as thrombocidin-2 (TC-2). These proteins migrate in an SDS gel as proteins with an apparent molecular weight of 5.5 and 6.5 kD, respectively (fig 5).

ii) Continuous AU-PAGE

Fractions eluted from the CM-sepharose column containing antibacterial protein (30 through 75) were pooled, lyophilized, and subjected to a second purification step utilizing continuous gel electrophoresis. Cylindrical gels (3,7x6 cm, 12.5% acrylamide, 3M urea, 5% acetic acid) were poured in a model 491 Prep Cell (BioRad, Veenendaal, The Netherlands) and polymerized at 37°C. Prerunning was at 200V for 2 h. Sample (max. 450 µl) was electrophorized at 40 mA. Protein was eluted in 5% acetic acid at 1 ml/min and collected in 5 ml fractions. Again, fractions were analyzed in two urea gels run in parallel, followed by staining or by an assay for antibacterial activity (fig 6). TC-1 and TC-2 could effectively be separated (fig 6a). Both proteins had considerable activity against E. coli ML35 (fig 6b). Purified TC-1 and TC-2 were lyophilized and redissolved in 0,01% acetic acid, and stored at -20°C until further analysis.

C. Structure of thrombocidin-1 and -2

Bactericidal proteins thrombocidin-1 and -2, purified from human blood platelets, were analyzed by MALDI and electrospray (ES) mass spectrometry. ES analysis of TC-1 (fig 7a) yielded a molecular weight of 7436,3 ± 1,3 Da. Analysis by MALDI (fig 8) also revealed a peak of this size, next to an additional number of peaks with M+1 of 7107.2, 7227.7 and 7602,0 Da. The molecular weights of these proteins can be explained by assuming that these proteins are C-terminal truncation products of NAP-2; the calculated molecular weights correspond well with the values experimentally determined (Table 1). These data suggest that TC-1 is a mixture of C-terminally truncated forms of NAP-2. The 7436 Da protein seems to be the main component. We designated this protein TC-1.

~~ES spectroscopy of TC-2 (fig 7b) yielded a molecular weight of 9100,5 ± 1,3. This value was~~

confirmed by MALDI-tof spectroscopy. In addition to TC-2, only one minor contamination was present (10081 Da, fig 9). Partial sequencing of TC-2 indicated that the N-terminus of TC-2 is identical to that of CTAP-III. Based on the mass-spectrometrical data (figs 7b and 9) however, it appears that the mass found experimentally was smaller than the mass of CTAP-III (table 1). This can be explained by assuming that TC-2 is truncated C-terminally and misses 2 amino acids compared to CTAP-III. Thrombocidins identified thus far are indicated in fig 1, together with the sequences of CTAP-III and NAP-2.

Sub A'

Table 1. Interpretation of mass-spectrometrical data of TC-1 and TC-2: comparison with CTAP-III

Component	Mol. weight (Da) MALDI/ES	Calc	Sequence of N-terminus	C-terminus
CTAP-III		9287,2	NLAKGKEESLDSDLYAELR....	.AGDESAD
TC-1a	7106,2	7105,8	AELR....	.AG
TC-1b	7226,7	7220,9	AELR....	.AGD
TC-1c	7436,3	7437,5	AELR....	.AGDES
TC-1d	7601,0	7600,7	YAELR....	.AGDES
TC-2	9100,5	9101,6	NLAKGKEESLDSDLYAELR....	.AGDES

Sub A2

EXAMPLE 2

Production of recombinant (r)CTAP-III, rNAP-2, rTC-1, rTC-1' and rTC-2.

From a human bone marrow CDNA library (Clontech, Palo Alto, USA) DNA coding for PBP was amplified in a PCR. 5'TATAGGATCCATGAGCCTCAGACTTGATACCACC-3'and 5'TATAGGATCCTCAATCAGCAGATTCATCACCTGCCAAT-3'were used as forward and reverse primers, respectively. BamHI restriction sites (underlined) were added to allow cloning in a suitable vector. A stop sequence (boldface) was included to allow proper transcription termination at the stage of protein expression. This PCR was performed using 2 ng of template DNA and Pfu DNA polymerase, which has proofreading capacity. The resulting product was of the expected size (400 bp). This product served as a template in a second PCR to produce the coding DNA of TC-1, TC-2, CTAP-III, NAP-2 and TC-1', a variant of TC-1 which lacks two C-terminal amino acids (Ala-Asp) and carries two additional N-terminal amino acids (Ala-Glu) (fig 2). These PCR products were cloned into expression vectors. For CTAP-III, NAP-2 and TC-1 the reverse primer was the same as the reverse primer described above. The forward primers were as follows:

for CTAP-III and TC-2:
5'GTGTAACATATGAACTTGGCGAAAGGCAAAGAG-3';
for NAP-2 and TC-1*:
5'GTGTAACATATGTATGCTGAACTCCGCTGCATG-3';
and for TC-1:
5'GTGTAACATATGTATCTCCGCTGCATGTGTATAAAG-3'.

NdeI restriction sites (underlined) were included to allow cloning into the vectors. The PCR products were digested with NdeI/BamHI and ligated into a pET9a (TC-2, CTAP-III and TC-1*) or a pET16b vector (NAP-2 and TC-1), linearized with NdeI and BamHI. The recombinant proteins produced using the pET9a vector carry an additional N-terminal methionine, and are therefore designated as rMTC-1* and rMTC-2 and rMCTAP. The recombinant proteins produced using the pET16 vector carry an N-terminal His-tag, plus a tyrosine (Y) residue, and therefore designated as rYTC-1 and rYNAP. Constructs were transformed to E. coli DH5α, and plated on LB/kanamycin (50 µg/ml) (pET9a) or LB/ampicillin (50 µg ml) (pET16b) plates. Sequencing of cloned DNA confirmed the correct sequence in the constructs. Plasmids containing the correct insert were isolated and transformed to BL21(DE3)lysE cells, and plated on LB plates supplemented with the proper antibiotics. Of each plate, single colonies were picked, grown and stored in glycerol broth at 70°C until further use. Cultures of BL21(lysE) cells transformed with the CTAP-III, NAP-2 TC-1 or TC-1* genes containing pET expression vector were grown in LB medium supplemented with the proper antibiotics. Growing cultures with OD_{660} of 0.3 were induced with IPTG (1 mM final concentration). After 3 hours of induction, cells were harvested by centrifugation (5 min, 5000 g) and lysed in 20mM Tris HCl, pH 8,2 containing 6M guanidine HCl. Cell debris was removed by centrifugation. Supernatants of rMCTAP and r-MTC producing cells were dialyzed against 50mM phosphate buffer, pH 7,0.

rMTC-1*, rMTC-2 and rMCTAP were purified by CM-sepharose cationexchange chromatography and continuous acid urea gel electrophoresis, as described for TC-1* and TC-2 in example 1. The N-terminal His-tag in rYTC-1 and rYNAP allowed purification of these proteins using a His binding resin (Novagen). Final purification was performed by continuous AU PAGE. The structures of the purified recombinant proteins were confirmed by MALDI and ES mass-spectrometry.

EXAMPLE 3

Antibacterial activity

The experimental set-up for testing antibacterial activity of thrombocidins was as follows. Bacteria from blood agar plates were grown overnight in tryptic soy broth (TSB), subcultured in fresh TSB and grown to log-phase in 2-3 hours. Bacteria were pelleted, washed once in 10 mM phosphate buffer (pH 7,0) + 1% TSB (v/v) and resuspended in the same medium to an OD_{620} of 0,1. This suspension was further diluted 200 (B. subtilis) or 500 times (E coli and S. aureus) to obtain suspensions containing 0,5-1 x 10^5 colony forming units (cfu)/ml. In a polypropylene microtiter plate a serial dilution series of the protein to be tested was prepared in 0,01% acetic acid. To 5 µl of every sample, 45 µl of bacterial suspension (0,5-1 x 10^5 cfu/ml) was added. The plate was incubated on a rotary shaker (400 rpm) at 37°C. After 2 hours, 0,5 and 10 µl samples were plated on blood agar plates. Bactericidal activity was calculated the next day after colony counting. All experiments were performed in duplicate.

Bactericidal activity of TC-1* and TC-2 was determined against E. coli ML35, S. aureus 42D and B. subtilis ATCC6633 in killing assays (fig 10).

In fig 10 it can be seen that TC-1* and TC-2 are bactericidal against a all three bacteria tested, and that TC-1* is the more active component.

Bactericidal activity of TC-2 was tested against a panel of other bacterial species. The same method was used as described above, except that 5% Brain Heart Infusion (BHI) (v/v in water) instead of phosphate buffer + TSB was used as a test medium and one concentration of TC-2 was tested, being 100 μg/ml. Bacteria tested were E. coli ML35, wild-type E.coli, S. aureus 42D, multi-resistant S. aureus (MRSA), multiresistant S. epidermis (MRSE), and S. sanguis J30 (fig 11). The MRSA and MRSE are as susceptible to TC-2 as S. aureus 42D, while S. sanguis J30 seems to be slightly less susceptible.

Analysis of TC-1 and TC-2 in a non-reducing tricine gel revealed that the migration of both peptides was retarded compared to their reduced forms. This indicated that TC-1 and TC-2 contain disulfide bridges. To investigate whether the disruption of these disulfide bridges influenced antibacterial activity, reduced TC-2, reduced and alkylated TC-2, and non-reduced TC-2 were analysed for antibacterial activity using an acid urea gel overlay system. All three forms of TC-2 were equally active (fig. 12), indicating that disulfide bridges are not needed for TC-2 antibacterial activity. TC-1 was treated in the same way and showed similar results.

In general, disulfide bonds of cationic antibacterial proteins are considered to be essential for their antibacterial activity (Selsted and Harwig, 1989, Yomogida et al, 1995, Harwig et al, 1996, Piers et al, 1993, Proudfoot et al, 1997 and Lindley et al, 1988). The fact that in TC-1 and TC-2 the disulfide bonds are not needed for antibacterial activity is an unexpected finding.

MBCs of rMTC-1*, rMTC-2 and rMCTAP were determined for a number of organisms (Table 2). It appeared that rMTC-1* and rMTC-2 are bactericidal for B. subtilis, although MBCs are somewhat higher than for the native proteins (fig 10). The MBC of rMTC-1* for E. coli (3,8 μM) is the same as the MBC of the native protein TC-

1* (fig 10), whereas the MBC of rMTC-1* for S. aureus (15μM) is approximately 2-fold higher than for TC-1* (fig 10). In contrast to this, rMCTAP was not bactericidal for E. coli, B. subtilis and S. aureus at concentrations up to 40 μM. Recombinant NAP-2 (obtained from Bachem, Switzerland) was tested against B. subtilis up to 7μM, but no killing was observed.

Table 2. MBCs (μM) of recombinant proteins for various bacteria.

	B. subtilis	E. coli	S. aureus
rMTC-1*	3,8	3,8	15
rMTC-2	7,5	15	>15
rMCTAP	>40	>30	>30
rNAP-2	>7		

Bactericidal activity of rYTC-1 has been tested in a liquid assay as has been done for the native TCs. In comparison to the native thrombocidins TC-1* and TC-2, rYTC-1 is equally active against B. subtilis, but is more active against S. aureus 42D, and E.coli ML35 (table 3, fig 10). Additional experiments showed that rYTC-1 is highly active against a large number of bacterial species and against Cryptococcus neoformans (Table 3).

Table 3. Microbial activity of rYTC-1

Organism	MBC (μM) of rYTC-1
E. coli 69187 (EPEC)	0,9
E. coli 72540 (EPEC)	0,4
E. coli (genta 0)	0,9
E. coli ML35	0,9
Pseudomonas aeruginosa	3,8
Neisseria meningitidis W135	1,9
Klebsiella	1,9
Bacillus subtilis ATCC6633	0,4
S. aureus 42 D	1,9
MRSA	1,9
S. Epidermis RP62	0,9
S. Epidermis AMC 43	0,6
S. Epidermis AMC 48	0,6
S. Epidermis AMC 77	0,6
S. Epidermis AMC 82	1,2
S. Epidermis AMC 89	0,6
MRSE	1,2
S. sanquis U108	1,9
S. sanquis J30	1,9
Cryptococcus neoformans	0,4

Bactericidal activity of rMTC-1* against S. aureus, E. coli, and B. subtilis was significantly lower than that of rYTC-1 (table 2 and 3). The marked difference in bactericidal activity between rMTC-1* vs rNAP-2, and between rMTC-2 vs rMCTAP (Table 3) shows that the 2 additional C-terminal amino acids, alanin (A) and aspartic acid (D) present in rNAP-2 and rMCTAP strongly reduce bactericidal activity. Although these C-terminal amino acids are also present in rYTC-1, this protein is more active than rMTC-1*, which lacks the 2 C-terminal amino acids. In fact, rYTC-1 had more potent antibacterial activity than any of the native or recombinant proteins and rYTC-1 was also more active (MBC 0.4 μM) against Cryptococcus neoformans than rMTC-1* (MBC 7.5 μM) and rMTC-2 (MBC 15 μM). This indicates, that the N-terminal His-tag-containing sequence of rYTC-1 is involved in enhanced bactericidal activity, which has not been demonstrated before.

FIGURE LEGENDS

Figure 1: Sequences of thrombocidins and related proteins.

Figure 2: Recombinant proteins produced. Boxes indicate antimicrobial activity enhancing sequence (Histag).

Figure 3: Analysis of CM-sepharose purified platelet granular antibacterial protein. Selected fractions (as indicated) were run on AU-gels followed by silverstaining.
cav: crude granule extract (cavitate), starting material for the purification.

Figure 4: Analysis of CM-sepharose purified platelet granular antibacterial protein. Selected fractions (as indicated) were run on AU-gels followed by an ovelay using E. coli as a test organism.
cav: crude granular extract (cavitate), starting material for the purification.

Figure 5: Analysis of CM-sepharose purified platelet granular protein by tricine SDS-electrophoresis. Silverstained gel. Selected fractions (compare fig. 3) were anlayzed.

Figure 6: Analysis of AU-PAGE purified platelet granular antibacterial protein. Selected fractions (as indicated) were run on AU-gels followed by silverstaining (A) or followed by an overlay using E. coli as a test organism (B).
cav: crude granular extract (cavitate); CM: pooled active fractions (30-75) eluted from a CM-sepharose column.

Figure 7: Electrospray mass spectrometrical analyses of TC-1 (A) and TC-2 (B).

Figure 8: MALDI-tof mass spectrometrical analysis of TC-1.

Figure 9: MALDI-tof mass spectrometrical analysis of TC-2.

Figure 10: Antibacterial activity of TC-1 (top panel) and TC-2 (bottom panel) against E. coli ML35, S. aureus 42D, and B. subtilis ATCC6633 (0,5-1 x 10^5 cfu/ml) were incubated in the presence of serially diluted TC (concentrations are indicated). After 2 hours of incubation bacteria were plated and survival was determined by colony counting. Medium: 10 mM phosphate buffer pH 7,0 + 1% TSB. Of TC-2 0,3 and 0,7 μM were not tested against S. aureus and E. coli.

Figure 11: Killing of bacteria (1-2 x 10^5 cfu/ml) by TC-2 after 2 hrs incubation.

Figure 12: Antibacterial activity of TC-2 and reduced TC-2. Panel A: silverstained acid urea gel. Panel B: Overlay of acid urea gel (test organism: E. coli). Lanes 1: TC-2; Lanes 2: reduced and carboxymethylated TC-2. Lanes 3: TC-2 reduced by ß-mercaptoethanol treatment. Each lane contains approximately 3 μg of protein.

Figure 13: Three-dimensional structure of CXC chemokines.

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